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Disruption of plastid acyl:acyl carrier protein synthetases increases medium chain fatty acid accumulation in seeds of transgenic Arabidopsis

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ABSTRACT

Engineering transgenic plants that accumulate high levels of medium-chain fatty acids (MCFA) has been least successful for shorter chain lengths (e.g., C8). We demonstrate that one limitation is the activity of acyl-ACP synthetase (AAE) that re-activates fatty acids released by acyl-ACP thioesterases. Seed expression of *Cuphea pulcherrima* FATB acyl-ACP thioesterase in a double mutant lacking AAE15/16 increased 8:0 accumulation almost 2-fold compared to expression in wild type. These results also provide an *in planta* demonstration that AAE enzymes participate not only in activation of exogenously added MCFA but also in activation of MCFA synthesized in plastids.

1. Introduction

In plants, almost all *de novo* fatty acid synthesis takes place in the plastid stroma [1]. Plastidial elongation of acyl chains is terminated by the activity of acyl-ACP thioesterases, which catalyze the hydrolysis of the fatty acid-ACP thioester bond. These enzymes are therefore major determinants of the chain lengths of fatty acids produced in the plastid. Plants express two types of acyl-ACP thioesterases, FatA and FatB, which in most plants are responsible for release of 18:1 and 16:0, respectively [2].

Some plant species accumulate high levels (>50%) of medium chain fatty acids (MCFA, 6–14 carbons long) in their seed oil and in some cases up to 95% (e.g., *Cuphea pulcherrima*). These plants express specialized FATB acyl-ACP thioesterases that have evolved specificity for hydrolysis of medium chain length acyl-ACP substrates. MCFAs have valuable applications as free acids, alcohols and other feedstocks for the chemical industry. For example, MCFA esters are used as components in plasticizers, lubricants, surfactants, perfumes and flavoring [3,4]. Furthermore, oils with MCFA have lower caloric content and are more rapidly absorbed in the small intestine in animals. 12:0 and 14:0 FA are available from coconut and oil palm, both of which are species limited to cultivation in tropical climates. Thus, there are substantial economic and nutritional reasons for developing temperate crops with high levels of MCFA and this has been the target of a number of metabolic engineering efforts [5–7]. The most successful example is the accumulation of 58% mol% 12:0 in *Brassica napus*[8]. Unlike lauric (12:0), octanoic (8:0) and decanoic (10:0) acids are not available from current commercial oil crops. Large scale renewable production of octanoic would be particularly valuable because of several applications including in plasticizers (as octanol), and potentially as fungible biofuels. *Cuphea hookeriana* accumulates up to 75% 8:0 plus 10:0 in seed oil [9]. Transformation of *B. napus* with the FATB2 acyl-ACP thioesterase from *C. hookeriana* resulted in up to 35 mol% of 8:0/10:0 in seeds [10,11]. Thus, compared to *C. hookeriana* the level of 8:0 or 10:0 achieved in transgenic plants is substantially lower.

Several mechanism have been proposed to explain why transgenic plants are not able to accumulate levels of MCFA as high as native species. These include low enzyme specificity of downstream enzymes needed for MCFA incorporation, β-oxidation and disruption of membrane integrity [12]. Laurate is poorly incorporated into the *sn-2* position of TAG [13]. Poor incorporation by acyltransferase or other enzymes may subsequently lead to diversion of MCFA into β-oxidation and the turnover of lauric acid in

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transgenic *B. napus* developing seeds [14]. This limitation was partly overcome by co-expression of a coconut lysophosphatidic acid acyl transferase (LPAAT) together with *Uc*FATB in *B. napus* which resulted in increased 12:0 incorporation into TAG [8].

An additional less considered mechanism that may limit MCFA accumulation is that fatty acids released by the FATB acyl-ACP thioesterases are re-activated by plastidial acyl-ACP synthetase (AAE) reactions, thereby allowing their further elongation. Such a mechanism might be related to earlier studies where it was observed that medium-chain FA added to plant tissues are elongated to 16 and 18 carbon chain-lengths [15–17]. However, the enzymes or pathways responsible for this metabolism were unknown until Koo et al. [18] found that a mutant line disrupted in At4g14070 (AAE15) had an 80% reduction in elongation of exogenously supplied MCFA [18]. This gene and a closely related homolog, At3g23790 (AAE16) encode plastid-localized "acyl activating enzymes" that are members of a gene family encoding 63 proteins with AMP binding domains [19]. Because AAE are able to reactivate fatty acids of both medium and longer chain lengths, it is possible that they play a role for plants to cope with unintended hydrolysis of medium chain acyl-ACP hydrolysis [18] and/or are involved in acyl editing [20]. In this study we used the *aae 15/16* double mutant to investigate if reactivation of MCFA to form acyl-ACP reduces the ability of transgenic plants to accumulate MCFA in seed oil. Additionally, our results address whether AAE15/16 are involved in activation of plastid synthesized MCFA as well as those supplied exogenously to plant tissues.

2. Materials and methods

2.1. Plant growth

Arabidopsis varieties WS and *aae 15/16* were grown in soil at 120–150 μEs in 16/8h and 23/19C day/night cycle. The double mutant *aae 15/16* was generated by the single mutants *aae 15* and *aae 16* both in WS ecotype, as descripted in more detail in Koo et al. (2005) [18].

2.1.1. C. pulcherrima gene amplification and plant expression vector construction

Total RNA was isolated from frozen *C. pulcherrima* developing green seeds according to the method described by Suzuki et al. [21]. Candidate *C. pulcherrima* (*Cpu*) *FATB* thioesterase genes were identified among *C. pulcherrima* 454 sequences, through sequence homology to the *Arabidopsis thaliana FATB* (GI: 837372) gene. The *CpuFATB3* gene was amplified by PCR from *C. pulcherrima* developing seed cDNA using the following oligonucleotide primers with added *EcoRI* and *Xba*I restriction sites: 5′-TACG*AATT*CATGGTGGCTGCT GCAGCA-3′ and 5′-TAG*TCTAGA*CTAAGAGACCGAGTTTCCA-3′. The EcoRI and *Xba*I digested fragment containing the *CpuFATB3* gene was ligated into corresponding sites of the pBinGlyRed3 binary expression vector. pBinGlyRed3 contains the strong seed-specific soybean glycinin promoter and a DsRed marker gene, as described in Zhang et al. [22].

The *Umbellularia californica* (*UcFATB*, GI: 170555) thioesterase gene was codon optimized for expression in *A. thaliana* and synthesized with flanking *EcoRI* and *Xho*I restriction sites to facilitate directional cloning into the pBinGlyRed3 binary vector (GenScript, Piscataway, NJ, USA). *C. hookeriana ChFATB2* (GI: 1292905) and *Cuphea palustris CpFATB2* (GI: 1215719) clones were graciously donated by Jonathan Napier's group (Rothamsted Research, Harpenden, Herts, UK). Subsequently, *Eco*RI/*Sma*I and *Eco*RI/*Xho*I flanking fragments of *ChFATB2* and *CpFATB2*, respectively, were ligated into corresponding sites of the pBinGlyRed3 vector.

2.2. Plant transformation

Plasmids with *Cpu*FATB3, *Ch*FATB2, *Uc*FATB or *Cp*FATB2 acyl-ACP thioesterase genes controlled by the seed specific promoter glycinin were transformed into WS and *aae 15/16* background by floral dip using *Agrobacterium tumefaciens* [23]. Positive transformants were identified by DsRed fluorescence of seeds [24]. Tl, T2 and T3 seeds of wild type and *aae 15/16* transformants were selected on the same criteria to ensure that changes in acyl chain composition is a result of plant background and thioesterase and not a selection artifact introduced during the screening process. Homozygous T3 seeds were used for FAME composition, TAG molecular species and seed weight analysis. For each thioesterase in WT or *aae 15/16* background, seeds from 5 to 18 independent homozygous transgenic lines were analyzed.

2.3. Lipid extraction

100 seeds of each line were ground in 1 mL of isopropanol with a Polytron (PT 10-35 GT, Kinematica) and including 50 nmol tri15:0TAG and 50 nmol of tri9:0TAG as internal standards. Lipids were extracted according to [25]. TAGs were purified from lipid extracts using solid phase extraction (SPE). A glass–wool plugged Pasteur pipette was filled with 0.8 g of Silica gel 70–270, 60 Å and was preconditioned with 4 vol of $CHCl_3$:MeOH (99:1). Samples were dissolved in 500 μL toluene and loaded onto the SPE column. TAGs were eluted with 4 vol of $CHCl_3$:MeOH (99:1). TAGs were analyzed as intact TAG molecules by direct infusion electrospray ionization mass spectrometry (ESI-MS) and as fatty acid methyl esters (FAMEs) by gas liquid chromatography (GLC) equipped with a flame-ionization detector (FID). For ESI-MS analysis 5% of the lipid sample was dried under nitrogen gas and resuspended in 500 μL of isopropanol containing 10 μM tri13:0TAG and 10 mM NH_4Ac.

2.4. FAME analysis of medium and long acyl chains

TAG samples were dried under nitrogen gas and dissolved in 150 μL toluene and 1 mL of 5% H_2SO_4 in methanol and heated for 90 min at 70 °C in tightly capped tubes. Tubes were allowed to reach room temp and then 0.5 mL of 0.9% NaCl and 0.5 mL Hexane was added. To avoid losses due to the volatility of medium-chain length FAMEs, precautions were taken during transmethylation procedures and GC analysis. Following phase separation the organic phase was transferred without evaporation directly to a GC vial. FAMEs were separated on a DB-23 capillary column 30 m × 0.25 mm ID, 0.25 μm film thickness using helium as carrier gas at a constant flow of 1.5 mL min^{-1}. To ensure resolution of medium-chain FAMES from the solvent peak, a lower initial oven temperature of 115 °C for 3 min was used, then increased to 230 °C at 5 °C min^{-1} and maintained for 1 min. Injector and detector temperatures were maintained at 250 °C throughout the analysis.

2.5. ESI-MS analysis

TAG analysis was preformed in positive ion mode as ammonium adducts according to [26] with 10 mM NH_4Ac in isopropanol as mobile phase. Signal to analyte is dependent on acyl chain lengths and number of double bonds. Calibration curves with tri8:0, tri9:0, tri10:0, tri12:0, tri15:0, tri16:0, tri17:0, tri18:0, tri18:1, tri18:2, tri18:3 and tri19:0 TAG were analyzed at six different concentrations (0.5, 1, 2, 5, 10 and 20 μM). Each calibration curve was spiked with 10 μM tri13:0TAG. Calibration factors were calculated based on number of carbon and number of double bonds in relation to tri13:0TAG signal. Signal from biological TAG molecular species were correlated to ESI-MS internal standard (tri13:0-TAG) and quantified using calibration factors from standard curve.

3. Results and discussion

3.1. Medium-chain fatty acid accumulation is higher in an acyl-ACP synthetase mutant

A primary aim of this study was to investigate whether plastid localized acyl-ACP synthetase is one factor that limits the accumulation of MCFAs in transgenic Arabidopsis seeds. We hypothesized that MCFAs released from acyl-ACP by transgenic acyl-ACP thioesterase would be less likely to re-enter plastidial fatty acid elongation in *aae15/16* double mutant (Ws ecotype). To test this hypothesis, four genes encoding FATB acyl-ACP thioesterases from *C. pulcherrima* (*Cpu*FATB3, Genbank accession number KC675178). *C. hookeriana* (*Ch*FATB2, Gene bank ID: 1292905), *U. californica* (*Uc*FATB, Gene bank ID: 170555) and *C. palustris* (*Cp*FATB2. Gene bank ID: 1215719) were selected to provide chain length specificities that range from 8:0 to 14:0. (Table 1). The four different acyl-ACP thioesterase constructs, driven by the seed specific soybean glycinin promoter, were transformed into Arabidopsis wild type (WS ecotype) and into the *aae 15/16* mutant background.

When expressed in the WS wild-type background, all four thioesterases resulted in accumulation of MCFAs (Fig. 1) with chain lengths generally consistent with the composition of seeds from the species from which the thioesterase originated (Fig. 2, Table 1) and with reported in vitro chain-length specificity for the different acyl-ACP thioesterases (Table 1). The most abundant MCFA in transgenic seeds of plants transformed with *Cpu*FATB3, *Ch*FATB2, *Uc*FATB and *Cp*FATB2 were 8:0, 10:0, 12:0 and 14:0, respectively (Fig. 2). The maximum levels of MCFA accumulation in transgenic seeds were 19% (8:0 + 10:0), 25% (10:0 + 8:0), 43% 12:0 and 42% 14:0 for *Cpu*FATB3, *Ch*FATB2, *Uc*FATB and *Cp*FATB2, respectively (Table 2). As expected there was considerable variability in MCFA levels between independent transgenic lines expressing the same construct, presumably due to positional effects (i.e., where in the genome the transgene was inserted) and number of transgene insertions [11,27] (Fig. 1).

When the same constructs were expressed in the *aae 15/16* mutant background, the level of MCFA in seed TAG was higher for three of the four thioesterases. For *Cpu*FATB3, the average 8:0 level increased almost 2-fold, from 4.8 to 9.2 mol% (different at *P*-value <0.0001) and the total MCFA (8:0 + 10:0) increased from 11.7 to 15.9 mol%. Although the mol% MCFA range of the independent transformants overlap, the increases attributable to expression in the *aae* 15/16 mutant background were statistically significant at $P = 0.01$; $P = 0.05$; and $P = 0.1$ for lines expressing *Cpu*FATB3, *Ch*FATB2 and *Uc*FATB, respectively (Fig. 1A–C).

With increasing chain length i.e., 14:0 > 12:0 > 10:0 > 8:0, seeds accumulated higher levels of MCFA (Fig. 2A–D) in both the WT and *aae 15/16* mutant background. This is possibly a consequence of the specificity of Arabidopsis enzymes involved in glycerolipid/TAG assembly that less easily accepts shorter chain lengths. Species that accumulate specific FA have apparently co-evolved a number of enzymes (e.g., acyl-CoA synthetase, GPAT, LPAAT and DGAT [28,29]) that accommodate these structures. For example, in safflower, the acyl-CoA synthetase activity in microsomes is 45-fold higher for 18:2 compared to 8:0 FA [30]. Thus, we can expect that Arabidopsis enzymes are better suited to use acyl chains with the longer FA normally found in Arabidopsis compared to MCFA produced by thioesterases not normally found in Arabidopsis. If not accepted by the transgenic host enzymes, the MCFA may become reactivated to acyl-ACP by AAE, elongated by fatty acid synthase, or alternatively undergo β-oxidation.

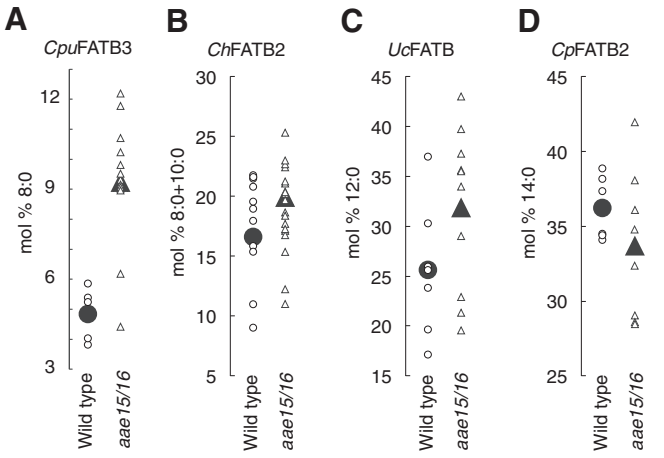

Fig. 1. Accumulation of medium chain fatty acids is increased in mutants of acyl-ACP synthetase. Open symbols represent independent transformation events expressing *Cpu*FATB3 (A), *Ch*FATB2 (B), *Uc*FATB (C) and *Cp*FATB2 (D). Solid symbols represent average mol% medium chain fatty acids in seed TAG. Circles and triangles represent wild type and *aae 15/16* background, respectively. Note that the *y*-axis do not start at zero. Students *t*-test: $P < 0.01$ (A), $P = 0.05$ (B), $P = 0.10$ (C) and $P = 0.27$ (D).

In addition to demonstrating that 8:0, 10:0 and 12:0 accumulation can be increased by expression in *aae 15/16* lines, these results establish that acyl-ACP synthetase enzymes activate not only exogenously added fatty acids [18], but also those produced *in planta*. Fig. 3 summarizes our model for the action for AAE15/16 in transgenic plants expressing *Cpu*FATB3

3.2. Long chain fatty acid composition is altered in plants expressing acyl-ACP thioesterases

With increasing levels of MCFA in transgenic seed, it is expected that the proportion of long chain fatty acids will be correspondingly reduced [10,11]. This was the case for both WT and *aae 15/16* plants that accumulate 12:0 and 14:0 MCFA, which show a decrease in all C18 FA (Fig. 2G and H). However, in seeds producing 8:0 and 10:0, the 18:1 mol% *increased* approximately 2-fold from 13 mol% in non-transformed lines to 27 mol% and 24 mol% in seeds transformed with *Cpu*FATB3 and *Ch*FATB2 (Fig. 2E and F). There was an approximately linear positive correlation between 8:0 and 10:0 levels and the increase in mol% 18:1 (Fig. 4A and B). All other long chain fatty acids (16:0, 18:2, 18:3) correlated negatively or showed no correlation to MCFA accumulation. In contrast, transgenic Arabidopsis that primarily accumulate 12:0 and 14:0 (both *Uc*FATB and *Cpu*FATB2) have a negative correlation between MCFA and 18:1 (and 18:2/18:3) (Fig. 4C and D).

Decreased levels of monounsaturated 18:1, and increases in 18:2 and 18:3 is commonly observed in plants and other organisms grown at low temperatures and has been attributed as an

Table 1
Medium chain fatty acid composition, genes and substrate specificity used in this study.

Species	MCFA	Gene name	Substrate specificity	Reference
Cuphea pulcherrima	94% 8:0	*Cpu*FATB3	Not reported	[9]
Cuphea hookeriana	50% 8:0, 25% 10:0	*Ch*FATB2	8:0-ACP, 10:0-ACP	[10]
Umbellularia californica	20% 10:0, 70% 12:0	*UcFATB*	12:0-ACP	[5,35]
Cuphea palustris	20% 8:0, 65% 14:0	*Cp*FATB2	14:0-ACP, 16:0-ACP	[33]

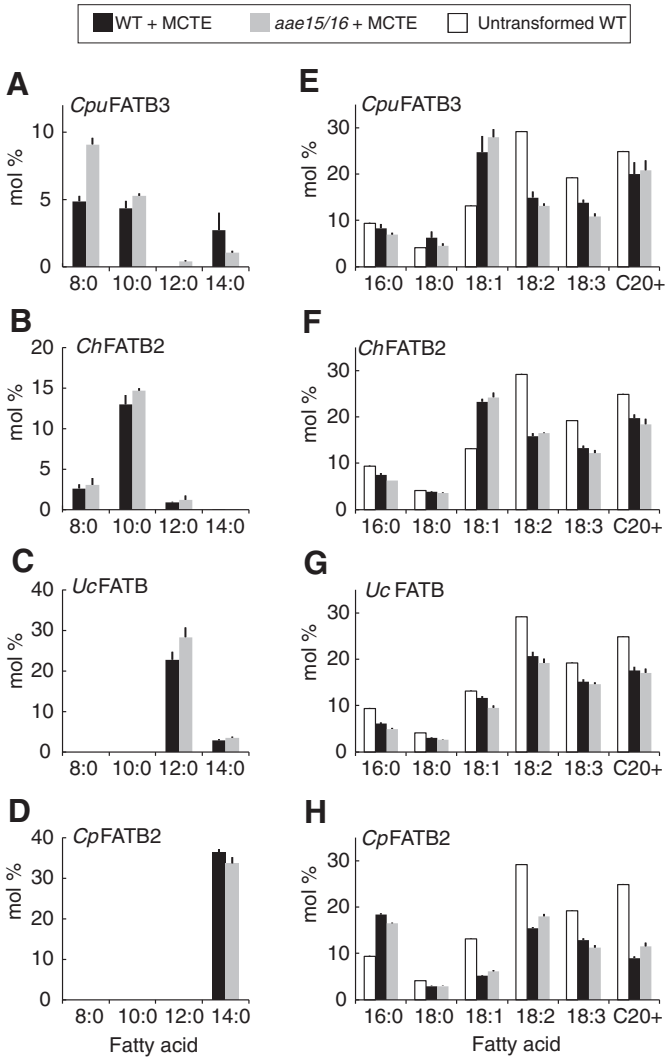

Fig. 2. Fatty acid composition in seed TAG of wild type (black bars) and *aae 15/16* plants (grey bars) expressing *Cpu*FATB3 (A, E), *Ch*FATB2 (B, F) *Uc*FATB (C, G) and *Cp*FATB2 (D, H). White bars represent untransformed wild type. Values represent average ± SE. No medium chain fatty acids were detected in wild type or *aae 15/16* backgrounds. There is no difference in fatty composition between wild type and *aae 15/16* background (Supplemental Table I).

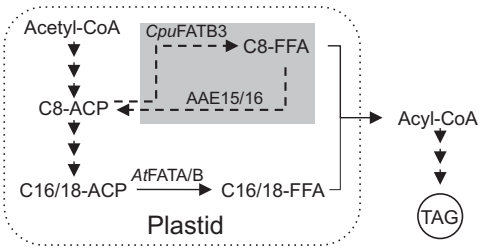

Fig. 3. Release and reactivation of C8 free fatty acid in transgenic Arabidopsis expressing *Cpu*FATB3. C8 FFA is released from C8-ACP by *Cpu*FATB3. Through acyl:acyl carrier protein synthetases (AAE15/16) a portion of C8-FFA is reactivated to C8-ACP and is further elongated to C16/18. In the *aae 15/16* mutant, reactivation is reduced, allowing more C8 FFA to be exported out of the plastid, and incorporated into TAG.

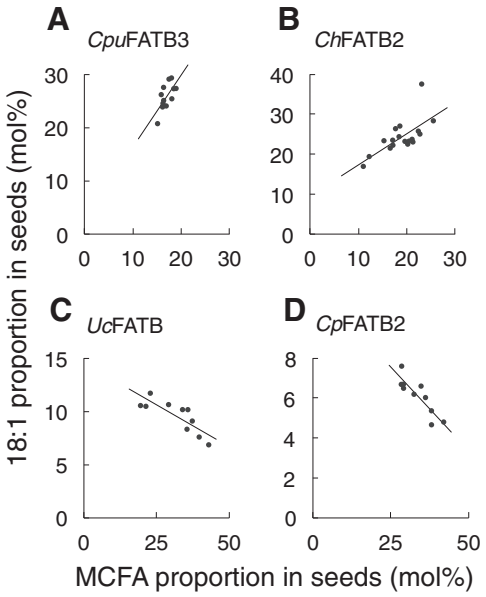

Fig. 4. Relationship between medium chain fatty acids and 18:1 content in *aae 15/16* plants expressing *Cpu*FATB3 (A), *Ch*FATB2 (B) *Uc*FATB (C) and *Cp*FATB2 (D). Each data point represents one individual transformation event.

Table 2
Measured vs predicted level of tri-MCFA triacylglycerols. Data are from transgenic lines with the highest content of MCFA. Measured content are from ESI-MS analysis and random values are calculated based on fatty acid composition assuming equal use of all fatty acid for three positions.

Acyl-ACP thioesterase	MCFA (mol%)	tri-MCFA TAG (mol%)	
	GC-FID	Measured	Predicted if random
*Cpu*FATB3	18.6	*nd*	0.6
*Ch*FATB2	25.4	*nd*	1.6
*Uc*FATB	43.0	3.8	8.0
*Cp*FATB2	41.9	4.6	7.4

nd: not detected.

adaptive mechanism to adjust membrane fluidity [31]. This response is also observed in the TAG content of oilseeds [32]. Therefore, one explanation for an increase in 18:1 and decrease in 18:2 and 18:3 in plants that accumulate 8:0 and 10:0 is that transgenic MCTE plants modulate fatty acid desaturation in response to the greater fluidity of membranes (or TAG) containing 8:0 and/or 10:0. An additional possibility is that the Arabidopsis FAD2 is less able to desaturate 18:1 to 18:2 when paired with 8:0 or 10:0 on PC.

3.3. Non-random distribution of MCFA in TAG molecular species

In naturally occurring triacylglycerols, unsaturated fatty acids are more abundant at *sn-2* of glycerol, whereas saturated, or unusual FAs are generally excluded from *sn-2*. As a result, there is an under-representation of species with the same FA on three positions compared to that calculated from a random distribution. To further understand the metabolism of MCFA in transgenic Arabidopsis seeds, we used ESI-MS to analyze the molecular species of TAG from Arabidopsis expressing the FATB acyl-ACP thioesterases. Fig. 5 presents mass spectra of TAG species from seeds expressing the four transgenes in the *aae 15/16* background. TAG species with at least one MCFA represent 48%, 51%, 40% and 23% in plants expressing *Cpu*FATB3, *Ch*FATB2, *Uc*FATB and *Cp*FATB2, respectively (peaks in shaded green area in Fig. 5).

As noted above, with increased levels of 8:0 and 10:0 MCFA there is a concomitant increase of 18:1 from 13 to 27 mol% in the FA composition (Fig. 2E and F). To further investigate the 8:0/10:0-to-18:1 connection we examined TAG molecular species with one or two MCFA paired with two or one 18:1 species. This analysis

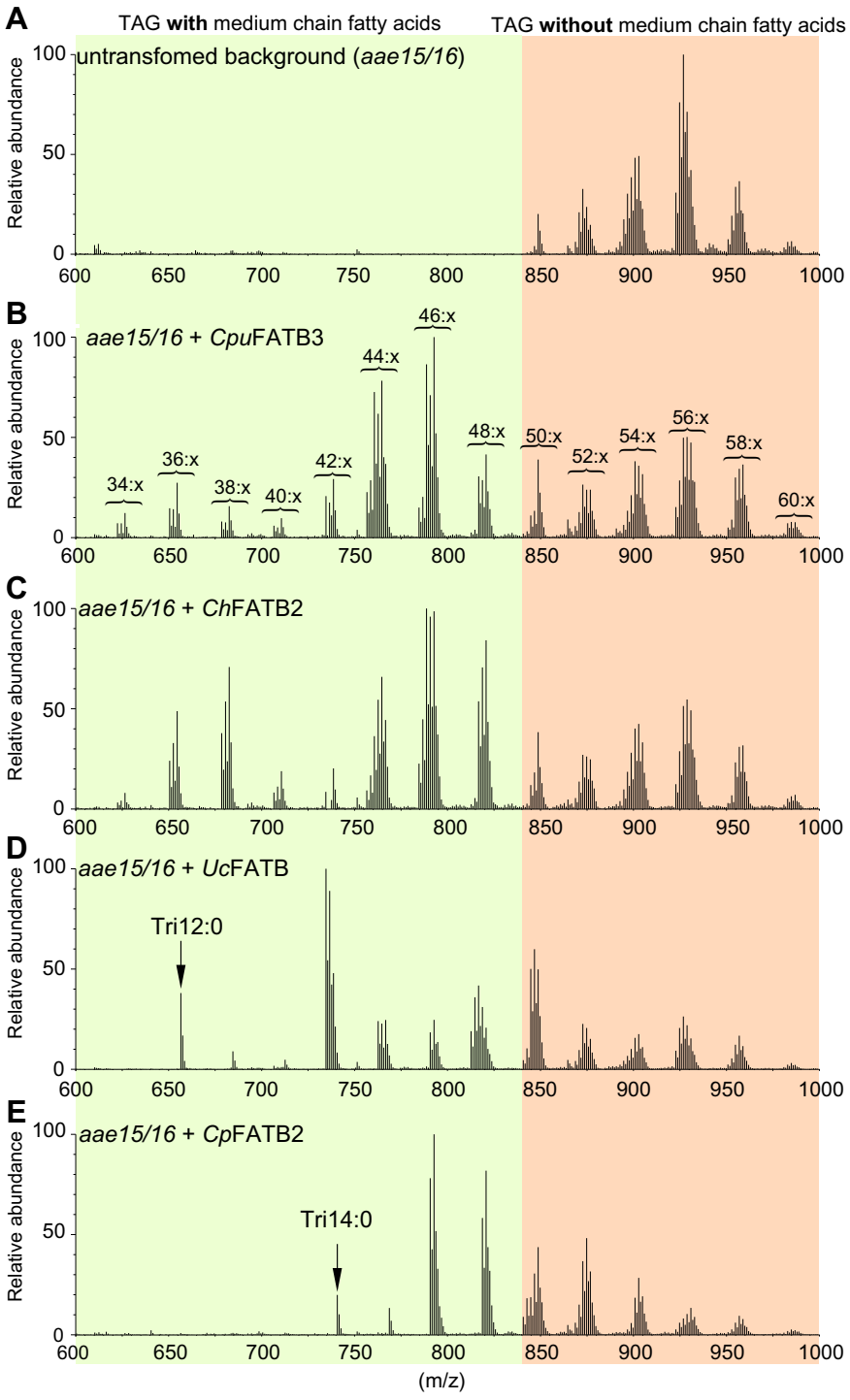

Fig. 5. Electrospray mass spectroscopy of TAG isolated from *aae 15/16* expressing no medium acyl chain thioesterase (A), *Cpu*FATB3 (B), *Ch*FATB2 (C) *Uc*FATB (D) and *Cp*FATB2 (E). Nomenclature: 54:*x*, for example, denotes TAG with 54 acyl carbons and *x* number of total double bond.

revealed that TAG species with one or two 18:1 paired with two or one 8:0/10:0 FA have a positive correlation to the level of MCFA in *Cpu*FATB3 and *Ch*FATB2 (Fig. 6). Thus, our evidence indicates that 8:0 and 10:0 MCFA are preferably paired with 18:1 FA compared to 18:2 or 18:3 in seed TAG in transgenic Arabidopsis. This phenomenon was not observed in the *Uc*FATB and *Cp*FATB2 transgenics producing 12:0 and 14:0.

Additional evidence for mechanisms that specify the molecular species of TAG can be found in the difference between measured and predicated levels of tri-MCFA-TAG species (Table 2). The theoretical level of tri-MCFA-TAG, calculated based on a random distribution of MCFA is higher than the measured value in all cases. For transgenic Arabidopsis expressing *Uc*FATB or *Cp*FATB2, tri12:0 or tri14:0 represented 3.8 and 4.6 whereas if randomly distributed would be 8% and 7.4%. No TAG species with MCFA at all three positions were detected in transgenic Arabidopsis expressing *Cpu*FATB3 or *Cpu*FATB2 (Fig. 5B and C). Previous analysis of *C. palustris* TAG reported similar phenomenon. If distributed randomly tri-14:0 would represent 12% of all TAG species, but only trace amounts were detected [33]. Thus mechanisms that restrict

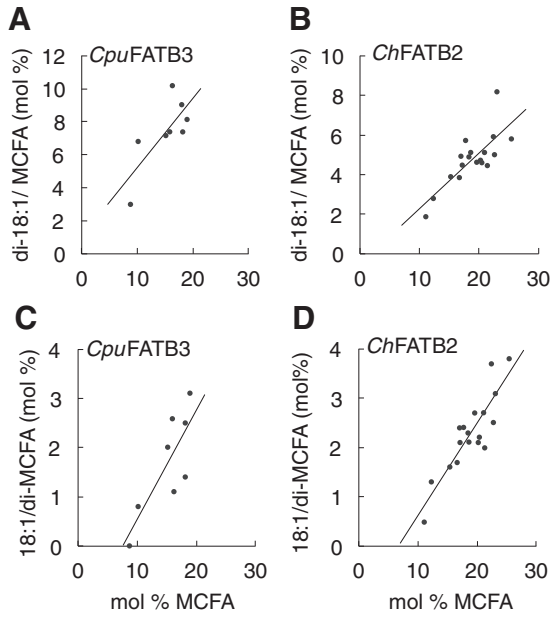

Fig. 6. Correlation between MCFA and (A, B) di-18:1/MCFA and (c, d) 18:1/di-MCFA. Solid circles represent independent transformation events from *aae 15/16* expressing *Cpu*FATB3 (A, C) and *Ch*FATB2 (B, D).

accumulation of TAG species with three MCFA are present not only in native species producing MCFA but also in transgenic plants coping with non-native fatty acid synthesis.

In summary, our results provide two major conclusions: (1) AAE enzymes participate in activation of MCFA synthesized in plastids, in addition to exogenously added FA and (2) it is possible to increase the accumulation of MCFA TAG in seed oils by limiting acyl-ACP synthetase activity. The mol% of 8:0 in seed TAG was increased by almost 2-fold in *aae* 15/16 seeds expressing *Cpu*FATB3 acyl-ACP thioesterases. The fold increase of 8:0 is higher than the enhancement observed for ricinoleic when castor DGAT2 is co-expressed with the FAH hydroxylase [34] or the enhancement in laurate levels when coconut LPAAT is co-expressed with *Uc*FATB [8]. Because of its potential applications and low availability from natural sources, 8:0 production in high-yielding oilseed crops can potentially be a valuable commodity. This study illustrates that AAE reduction is one approach that may be a useful adjunct to other strategies to increase MCFA, particularly 8:0, accumulation in crops.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.02.021.

References

[1] Ohlrogge, J.B., Kuhn, D.N. and Stumpf, P.K. (1979) Subcellular localization of acyl carrier protein in leaf protoplasts of *Spinacia oleracea*. Proc. Natl. Acad. Sci. USA 76, 1194–1198.
[2] Salas, J.J. and Ohlrogge, J.B. (2002) Characterization of substrate specificity of plant FatA and FatB acyl-ACP thioesterases. Arch. Biochem. Biophys. 403, 25–34.
[3] Dyer, J.M., Stymne, S., Green, A.G. and Carlsson, A.S. (2008) High-value oils from plants. Plant J. 54, 640–655.
[4] Ohlrogge, J.B. (1994) Design of new plant products – engineering of fatty acid metabolism. Plant Physiol. 104, 821–826.
[5] Pollard, M.R., Anderson, L., Fan, C., Hawkins, D.J. and Davies, H.M. (1991) A specific acyl-ACP thioesterase implicated in medium-chain fatty acid production in immature cotyledons of *Umbellularia californica*. Arch. Biochem. Biophys. 284, 306–312.
[6] Voelker, T., Worrell, A., Anderson, L., Bleibaum, J., Fan, C., Hawkins, D., Radke, S. and Davies, H. (1992) Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. Science 257, 72–74.
[7] Dehesh, K. (2001) How can we genetically engineer oilseed crops to produce high levels of medium-chain fatty acids? Eur. J. Lipid. Sci. Technol. 103, 688–697.
[8] Knutzon, D.S., Hayes, T.R., Wyrick, A., Xiong, H., Maelor Davies, H. and Voelker, T.A. (1999) Lysophosphatidic acid acyltransferase from coconut endosperm mediates the insertion of laurate at the *sn-2* position of triacylglycerols in lauric rapeseed oil and can increase total laurate levels. Plant Physiol. 120, 739–746.
[9] Graham, S.A. and Kleiman, R. (1992) Composition of seed oils in some Latin American *Cuphea* (L ythraceae). Ind. Crops Prod. I, 31–34.
[10] Dehesh, K., Jones, A., Knutzon, D.S. and Voelker, T.A. (1996) Production of high levels of 8:0 and 10:0 fatty acids in transgenic canola by overexpression of *ChFatB2*, a thioesterase cDNA from *Cuphea hookeriana*. Plant J. 9, 167–172.
[11] Voelker, T.A., Hayes, T.R., Cranmer, A.M., Turner, J.C. and Davies, H.M. (1996) Genetic engineering of a quantitative trait – metabolic and genetic parameters influencing the accumulation of laurate in rapeseed. Plant J. 9, 229–241.
[12] Jaworski, J. and Cahoon, E.B. (2003) Industrial oils from transgenic plants. Curr. Opin. Plant Biol. 6, 178–184.
[13] Wiberg, E., Banas, A. and Stymne, S. (1997) Fatty acid distribution and lipid metabolism in developing seeds of laurate-producing rape (*Brassica napus* L.). Planta 203, 341–348.
[14] Eccleston, V.S. and Ohlrogge, J.B. (1998) Expression of lauroyl-acyl carrier protein thioesterase in *Brassica napus* seeds induces pathways for both fatty acid oxidation and biosynthesis and implies a set point for triacylglycerol accumulation. Plant Cell 10, 613–622.
[15] Kannangara, C.G., Jacobson, B.S. and Stumpf, P. (1973) Biosynthesis of a-linolenic acid in plants. Biochem. Biophys. Res. Commun. 52, 648–655.
[16] Terzaghi, W.B. (1986) A system for manipulating the membrane fatty acid composition of soybean cell cultures by adding tween-fatty acid esters to their growth medium: basic parameters and effects on cell growth. Plant Physiol. 82, 771.
[17] Thompson Jr., G.A. and Roughan, P.G. (1986) Spinach leaves desaturate exogenous [^{14}C] palmitate to hexadecatrienoate: evidence that de novo glycerolipid synthesis in chloroplasts can utilize free fatty acids imported from other cellular compartments. Plant Physiol. 82, 357.
[18] Koo, A.J.K., Fulda, M., Browse, J. and Ohlrogge, J.B. (2005) Identification of a plastid acyl–acyl carrier protein synthetase in Arabidopsis and its role in the activation and elongation of exogenous fatty acids. Plant J. 44, 620–632.
[19] Shockey, J.M., Fulda, M.S. and Browse, J. (2003) Arabidopsis contains a large superfamily of acyl-activating enzymes. Phylogenetic and biochemical analysis reveals a new class of acyl-coenzyme A synthetases. Plant Physol. 132, 1065–1076.
[20] Kaczmarzyk, D. and Fulda, M. (2010) Fatty acid activation in cyanobacteria mediated by acyl–acyl carrier protein synthetase enables fatty acid recycling. Plant Physiol. 152, 1598–1610.
[21] Suzuki, Y., Kawazu, T. and Koyama, H. (2004) Arabidopsis RNA isolation. Biotechniques 37, 542–544.
[22] Zhang, C., Cahoon, R.E., Hunter, S.C., Chen, M., Han, J. and Cahoon, E.B. (2012) Genetic and biochemical basis for alternative toutes of tocotrienol biosynthesis for enhanced vitamin E antioxidant production. Plant J., http://dx.doi.org/10.1111/tpj .12067.
[23] Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16, 735–743.
[24] Stuitje, A.R., Verbree, E.C., VanDerLinden, K.H., Mietkiewska, E.M., Nap, J.P. and Kneppers, T.J.A. (2003) Seed-expressed fluorescent proteins as versatile tools for easy (co) transformation and high-throughput functional genomics in Arabidopsis. Plant Biotech. J. 1, 301–309.
[25] Hara, A. and Radin, N.S. (1978) Lipid extraction of tissues with a low-toxicity solvent. Anal. Biochem. 90, 420–426.
[26] Han, X. and Gross, R.W. (2001) Quantitative analysis and molecular species fingerprinting of triacylglyceride molecular species directly from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry. Anal. Biochem. 295, 88–100.
[27] Wilson, C., Bellen, H.J. and Gehring, W.J. (1990) Position effects on eukaryotic gene expression. Annu. Rev. Cell. Biol. 6, 679–714.

[28] Cao, Y.-Z. and Huang, A.H.C. (1997) Acyl coenzyme A preference of diacylglycerol acyltransferase from the maturing seeds of *Cuphea*, Maize, Rapeseed, and Canola. Plant Physiol. 84, 762–765.
[29] Bafor, M., Jonsson, L., Stobart, A. and Stymne, S. (1990) Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations from the developing seeds of *Cuphea lanceolata*. Biochem. J. 272, 31.
[30] Ichihara, K.i., Takahashi, T. and Fujii, S. (1988) Diacylglycerol acyltransferase in maturing safflower seeds: its influences on the fatty acid composition of triacylglycerol and on the rate of triacylglycerol synthesis. Biochim. Biophys. Acta Lipid Metab. 958, 125–129.
[31] Murata, N. and Los, D.A. (1997) Membrane fluidity and temperature perception. Plant Physiol. 115, 875–879.
[32] Garces, R., Sarmiento, C. and Mancha, M. (1994) Oleate from triacylglycerols is desaturated in cold-induced developing sunflower (*Helianthus annum*) seeds. Planta 193, 473–477.
[33] Dehesh, K., Edwards, P., Hayes, T., Cranmer, A.M. and Fillatti, J. (1996) Two novel thioesterases are key determinants of the bimodal distribution of acyl chain length of *Cuphea palustris* seed oil. Plant Physiol. 110, 203–210.
[34] Burgal, J., Shockey, J., Lu, C., Dyer, J., Larson, T., Graham, I. and Browse, J. (2008) Metabolic engineering of hydroxy fatty acid production in plants: RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. Plant Biotech. J. 6, 819–831.
[35] Hopkins, C., Chisholm, M.J. and Prince, L. (1966) Fatty acids of *Lindera umbellata* and other Lauraceae seed oils. Lipids 1, 118–122.